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Biochemistry

Key interactions in antibody recognition of synthetic sweeteners: Crystal structures of NC6.8 Fab co-crystallized with high potency sweetener compound SC45647 and with TES.

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Running Title: Key interactions in antibody recognition of synthetic sweeteners

Abstract The complex crystal structures of the murine monoclonal IgG2b(?) antibody NC6.8 Fab fragment complexed with high-potency sweetener compound (SC45647) and non tasting high affinity antagonist TES have been determined. A comparative analysis with the structure of NC6.8 complexed with the super-potency sweetener NC174 reveals that the zwitterionic, tri-substituted guanidinium sweeteners as well as the zwitterionic antagonist TES interact with the same residues in the antigen binding pocket of NC6.8. In case of the non-sweetener TES, the interactions are largely indirectly mediated through a hydrogen bonded water network. The presence of a hydrophobic moiety as a major determinant for sweet taste has been confirmed, and its nature is likely a discriminator for super- versus high-potency sweeteners. Neither high-potency sweetener SC45657 nor non-sweet tasting TES induce any significant change in the NC6.8 Fab elbow angle, which excludes the necessity of ligand-induced allostery as a general requirement for ligand binding in NC6.8. A conserved water molecule mediating hydrogen binding to residues buried in the antigen binding pocket has been observed in all three NC6.8 complexes. Since the antigen binding pocket of NC6.8 can adopt to multiple ligands - including high affinity antagonists - it is important that structure guided sweetener design based on available complexes fully considers the exceptional structural adaptability of receptor-mimicking antibody models.

Keywords: antibody complex, taste receptor, sweetener, SC45647, antagonist, TES, glucophore

1. Introduction

Taste perception. The sensation of taste plays a critical role in the life and nutritional habits of humans and other organisms (Inoue et al., 2001). The perception of taste is elicited through the interaction of tastants with their receptors in the taste cells (Lindemann, 1996). It is widely believed that humans have four types of taste perception: sour, salty, bitter and sweet (Lindemann, 1996, Kinnamon and Cummings, 1992, Gilbertson et al., 2000). The chemical nature of tastants varies widely, and includes ions, small organic molecules, proteins, carbohydrates and amino acids. A tastant mediates taste perception by interacting with distinct

cell surface receptors that are expressed in a subset of taste cells. During the past few years, significant progress has been made in identification (Sainz et al., 2001), functional expression, and characterization of taste receptors (T1r1, T1r2 and T1r3) from mammals (Margolskee, 2002, Chandrashekar et al., 2000). While the amino acid sequences of these receptors are known, the key surface residues of the receptor responsible for biological activity have not yet been identified. To date, the structures of several sweet-tasting proteins have been determined by NMR (Caldwell et al., 1998, Spadaccini et al., 2001), but the functional groups which are responsible for taste perception have still to be determined, largely due to the lack of sequence and structural homology (Spadaccini et al., 2003) among the sweet tasting proteins. In view of the difficulty to obtain structures of sweeteners complexed to cell surface receptors, we have pursued an alternative approach, and we report the comparative analysis of crystal structures of synthetic sweetener and non-sweetener compounds complexed with murine monoclonal antibody (mAB) NC6.8.

Amtibody complex crystal structures as glucophore binding models. Several different models have been developed to describe the nature and topological arrangement of glucophores in an ideal sweet compound. A model for a receptor site with electrostatic potential, hydrogen bonding potential and hydrophobic interactions has been proposed to match the properties of low energy conformers of various sweeteners (Walters, 1991). The crystal structure of super-potency sweetener compound NC174, a zwitterionic, tri-substituted guanidine hapten, complexed with NC6.8 IgG2b(?) mAB raised against NC174, has been reported (Guddat et al., 1994). Interestingly, NC14.10, an isotype IgG2b(?) mAB, displayed a significantly different binding mode against the same hapten, attesting to the structural diversity in antigen recognition by immunoglobulins (Guddat et al., 2000). A comprehensive summary of the monoclonal antibody libraries that recognize super-sweeteners has been published (Anchin et al., 1997).

To further investigate the structural basis for taste perception, we analyzed the co-crystal structures of the NC6.8 Fab fragment, serving as a structural mimic for the elusive taste receptor, complexed with a structurally related high-potency sweetener SC45647 (Nofre et al., 1990) and with non-sweet tasting TES (N-[tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid). The analysis of super-potency (NC174, 200,000 times sweeter than 2% sucrose) and high-potency sweeteners (SC45647, 28,000 times sweeter) versus a non-sweetening compound allows to

identify key molecular interactions specifying the structure-activity relationship of zwitterionic sweet tasting molecules (figure 1). The crystal structures show that both sweetener compounds as well as TES bind in the same binding-pocket, but exhibit significant differences in binding patterns as well as in placement of the hydrophobic moieties. The structure comparison indicates that residues from CDR (Complementarily Determining Region) H2 and CDR H3 play major roles in the recognition of super-potency versus high-potency sweetener compounds. We were unable to complex low affinity sweeteners (NC90, NC24, NC274 and Aspartame) with NC6.8. Instead of these molecules, the TES buffer molecule was found in the antigen-binding pocket of NC6.8.

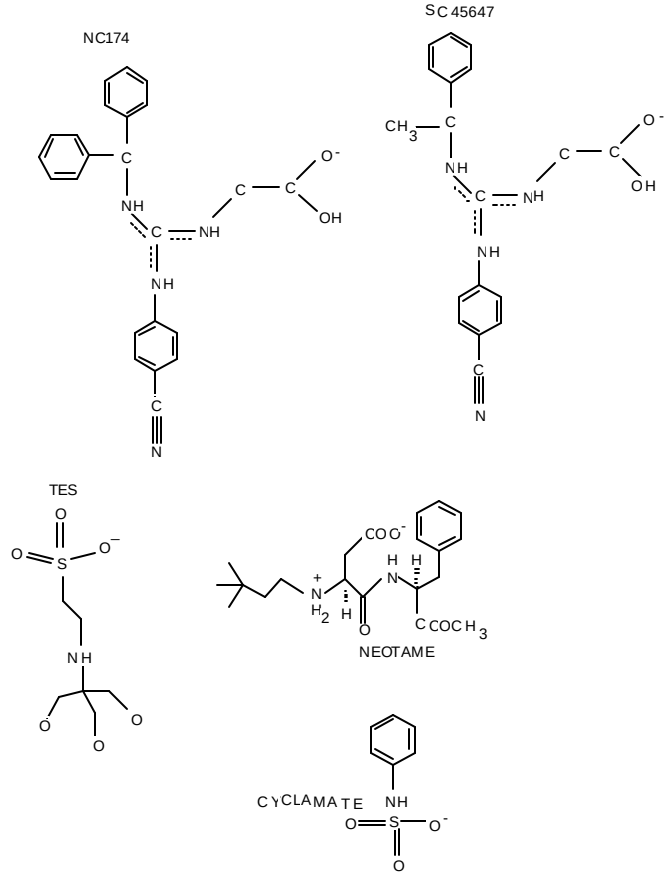

Figure 1. Sweet tasting molecules and TES. NC174 represents a super potency sweetener (200,000 times sweeter than 2% sucrose), SC45647 is a high-potency tastant (28,000 times sweeter), cyclamate and neotame represent medium potency sweeteners and TES is a non sweet tasting taste receptor antagonist.

2. Materials and Methods:

Purification of Antibodies

Murine NC6.8 IgG2b(?) antibodies were first precipitated from ascites fluid with 60% saturated ammonium sulfate precipitation as described (Liu et al., 1994). The precipitate was collected after centrifugation for 10 min at 10,000 g, then resuspended (2 mg/ml concentration) and dialyzed against 20 mM phosphate buffer (pH 7.2) overnight. The dialyzed sample was loaded onto a IgG column and washed thoroughly with phosphate. The bound antibodies were eluted with 0.1 M glycine-HCl buffer (pH 2.8), and the eluent immediately neutralized with 1M Tris pH 9.0. The affinity purified IgG was concentrated (6 mg/ml) using a Centriprep concentrator and digested by papain.

Papain (type III, 2 X crystallized from Sigma) was initially activated with cysteine as described (Parham et al., 1982). Proteolysis was carried out at 37ºC in 0.1 mM sodium acetate buffer pH 5.5 containing 3 mM EDTA and 50 mM cysteine. After 4 hrs proteolysis was stopped by addition of iodo acetamide (to alkylate the sulfhydryl groups) at a final concentration of 30 mM and the reaction products centrifuged for 30 min at 10,000 g. The supernatant was loaded onto a 2.5 x 100 cm high resolution Sephacryl S-200 (Pharmacia) column equilibrated at pH 8.0 with 25 mM Tris-HCl, 0.1 M NaCl, 1 mM NaN_3. The Fab eluted as the last peak, well separated from Fc fragments and uncleaved antibodies. Prior to crystallization trials, the purified antibody fragment solutions were concentrated to 40 mg/ml and dialyzed against 0.02 M TES buffer, pH 6.8 to a final concentration of 15 mg/ml.

Crystallization. NC6.8 Fab (10-15 mg/ml) was incubated (1 hour) with compound SC45647 in twofold molar excess. Initial crystallization conditions were screened in hanging drops (McPherson, 1982) using a sparse matrix kit (Crystal Screen I, Hampton Research, CA). Small crystals of the NC6.8-SC45647 complex grew in two days at 18°C from 4 µl sitting droplets consisting of a 1:1 mixture of stock NC6.8-SC45647 complex and a crystallization buffer containing 50 mM potassium hydrogen phosphate pH 9.2 containing 20% polyethylene glycol (PEG) 8000. Crystallization conditions were refined until crystals about 0.2 mm in size could be obtained in a reproducible manner within 3-4 days. Low-potency sweetener compounds NC90, NC24, NC274 and Aspartame were incubated in100-fold molar excess with the NC6.8 Fab fragment. Crystals grew under identical conditions and appeared within one to two weeks.

Data Collection. Crystals were harvested in Hampton cryo-loops and flash-cooled directly in the nitrogen cold stream (120K) after brief soaks in 2 µl mother liquor plus 2 µl 20% ethylene glycol as a cryoprotectant. Diffraction data were collected to 2.1 Å from a single crystal of NC6.8-SC45647 and to 1.7 Å data for NC6.8-TES at SBC beam line-19 at the Advanced Photo Source (APS) of the Argonne National Laboratory with a 4x4 module CCD detector. The data were reduced using *DENZO* (Otwinowski and Minor, 1997), and intensities were scaled in space group *C*2 with *SCALEPACK* (Otwinowski and Minor, 1997). Solvent content estimates (Matthews, 1968, Kantardjieff and Rupp, 2003) indicated the presence of one monomer in the asymmetric unit. Data collection statistics are summarized in Table 1.

Structure Determination of NC6.8-SC45647 and NC6.8-TES. Initial phases for the NC6.8-SC45647 complex and the TES-bound crystal form were obtained by molecular replacement using the program *CNS* (Brunger et al., 1998). The native NC6.8 Fab structure (1CGR) was used as a search model (Guddat et al., 1994). Separate cross-rotation functions for the variable and constant regions gave reliable solutions (data between 25 to 3.5 Å). The best solutions from the rotation search were used in the subsequent translation search carried out on a 0.25 Å grid, yielding strong and well-packing solutions.

The initial molecular replacement models were manually rebuilt with the program Xfit (McRee, 1999) into bias minimized, multiple averaged electron density maps obtained from the *SNW* (*Shake&wARP*) server (Reddy et al., 2003), and refined with *REFMAC5* (Murshudov et al., 1999). After repeated cycles of refinement and manual building, water molecules were manually added to the model using the *SNW* map. Clear electron density allowed unambiguous placement of the high affinity ligand SC45647 in the *SNW* maps (Figure 2). The low affinity ligand-Fab complex crystals revealed no density that was compatible with the incubated ligands. Instead, the binding sites contained a TES buffer molecule, which could be built unambiguously into the maps (Figure 2).

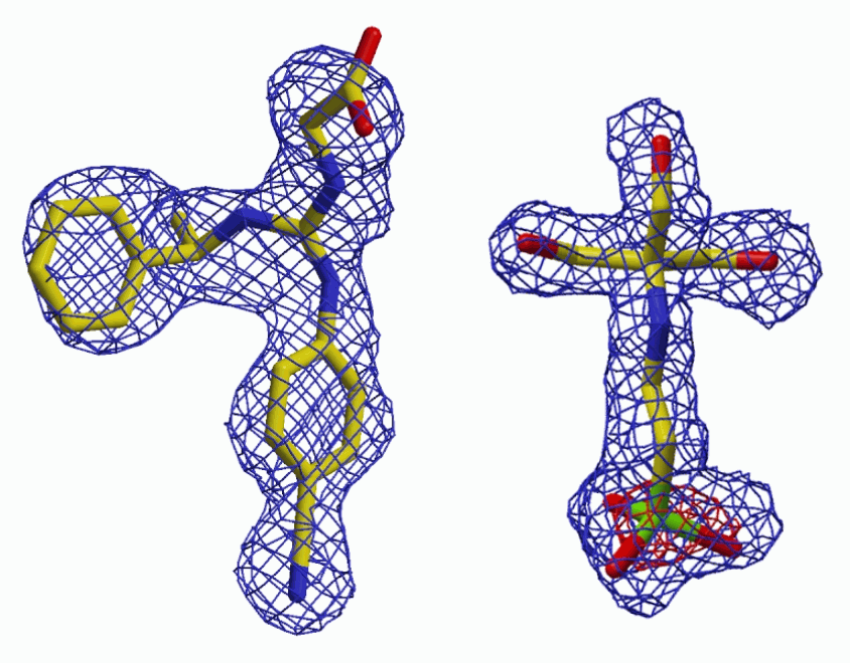

Figure 2. Electron density of ligands complexed with NC6.8 antibody. Electron density of *SNW* omit maps contoured at 1 s level (blue grid) and 5 s (red). Left panel: SC45647, right panel: TES. In both cases the ligands are oriented corresponding to figures 4a-c, pointing down into the antigenic binding pocket. Ligand molecules were omitted form the model before the *SNW* map generation. The blob feature in *XtalView* has been used to limit the display of the electron density to within 1.9 Å of the model. Figures created by *XtalView* (McRee, 1999) and rendered with *Raster3d* (Merritt and Bacon, 1997).

The completed models including SC45647 or TES, respectively, were submitted to a final round of refinement with *REFMAC5* (Table 1). The final SNW electron density maps were of high quality and density for residues L1-L214 of the L-chain (total of 219 residues) and H1-H158 and H164-H215 of the H-chain (also 219 residues) was clear; residues H159-H163 were less well defined. The antibody fragment residues were numbered following the numbering scheme for immunoglobulin by Kabat (Kabat, 1992).

3 Results

3.1. Overcall structure of the complex structures

The sweeteners NC174, SC45647 and non-sweet tasting TES buffer all bind in the same antigen binding pocket of the NC6.8 antibody fragment (figure 3). The binding pocket is formed by residues from CDR loops H1, H2, H3, L1 and L3 of the Fab fragment. The overall structure of NC6.8 is typical for IgG2b(?) antibody Fab fragments, and has been described previously (Guddat et al., 1994).

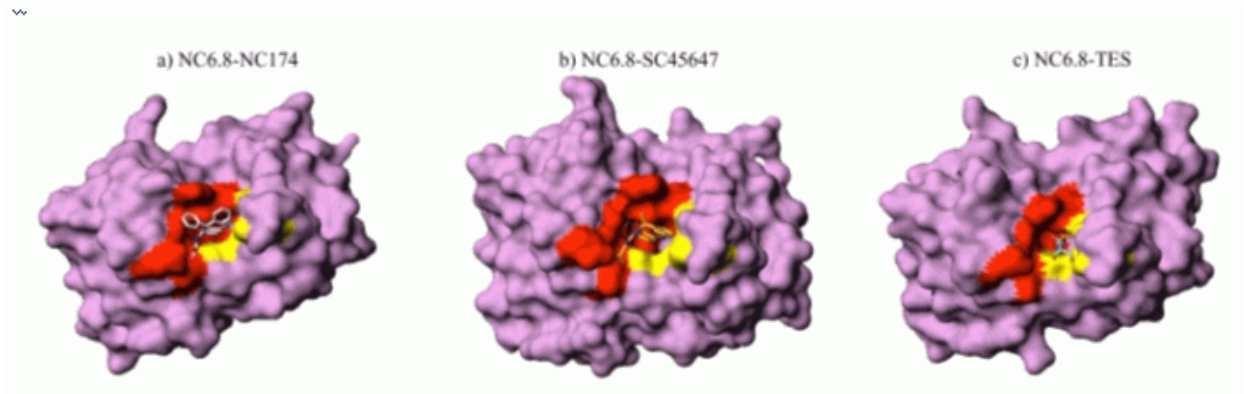

Figure 3. Surface representation of the Fab molecule showing the overall structure of the antigen binding pocket of the NC6.8 antibody complexed with a) super-potency sweetener NC174, b) high-potency sweetener SC45647 and c) non-sweet tasting TES. The light chain interacting residues are shown in yellow; heavy chain interacting residues are shown in red; ligand molecules are represented as ball and stick models.

3.2 Binding of high potency sweetener SC45647

The antigen-binding pocket of NC6.8 is highly complementary to high potency sweetener SC45647, burying 565 Å of the molecular surface. The ligand is clearly identifiable as the (R) stereoisomer with its chiral center at C8 (figure 2a). Most of the ligand atoms participate in hydrophobic interactions with residues from both the heavy chain and the light chain of NC6.8, with distinct hydrogen bonds to Glu 50H, Ser 97H and Arg 56H (figure 4a). The CN-group of SC45647, in practically the identical position as in the NC174 complex (figure 5), is sandwiched between Gly 91L and Tyr 96L and hydrogen bonded to Ser 97H. It interacts indirectly via a conserved water molecule with two light chain residues Ser89L and Tyr 36L, an interaction pattern also observed in the NC174 complex. The cyanophenyl ring of SC45647 participates in p-stacking with aromatic tyrosine Tyr 96L, with a ring-to-ring distance of ~ 3.5 Å typical for p-stacking. The unsubstituted phenyl ring of SC45647 is accommodated at the entrance of the

antigen binding pocket, and interacts through hydrophobic interactions primarily with Tyr 32L. The adjacent SC45647 methyl group exhibits weak hydrophobic interactions with the p-system of Tyr 96H. The tri-substituted guanidyl group of SC45647 p-stacks nicely with Trp 33H on one side, and has weak hydrogen bonds to the carboxyl group of Glu 50H and to Tyr 96H O?. On the other side, the guanidyl group forms weak hydrogen bonded contacts to Glu 50H and Try 96H. The SC45647 acetyl group is hydrogen bonded to Nd2 of Asn 58H and Ne of the guanidyl group of Arg 56H, again in a conformation and interaction pattern similar to NC174 (figure 4c). Electron density however does indicate that a minor conformation consistent with the alternate (split) conformation of Arg 56H may exist.

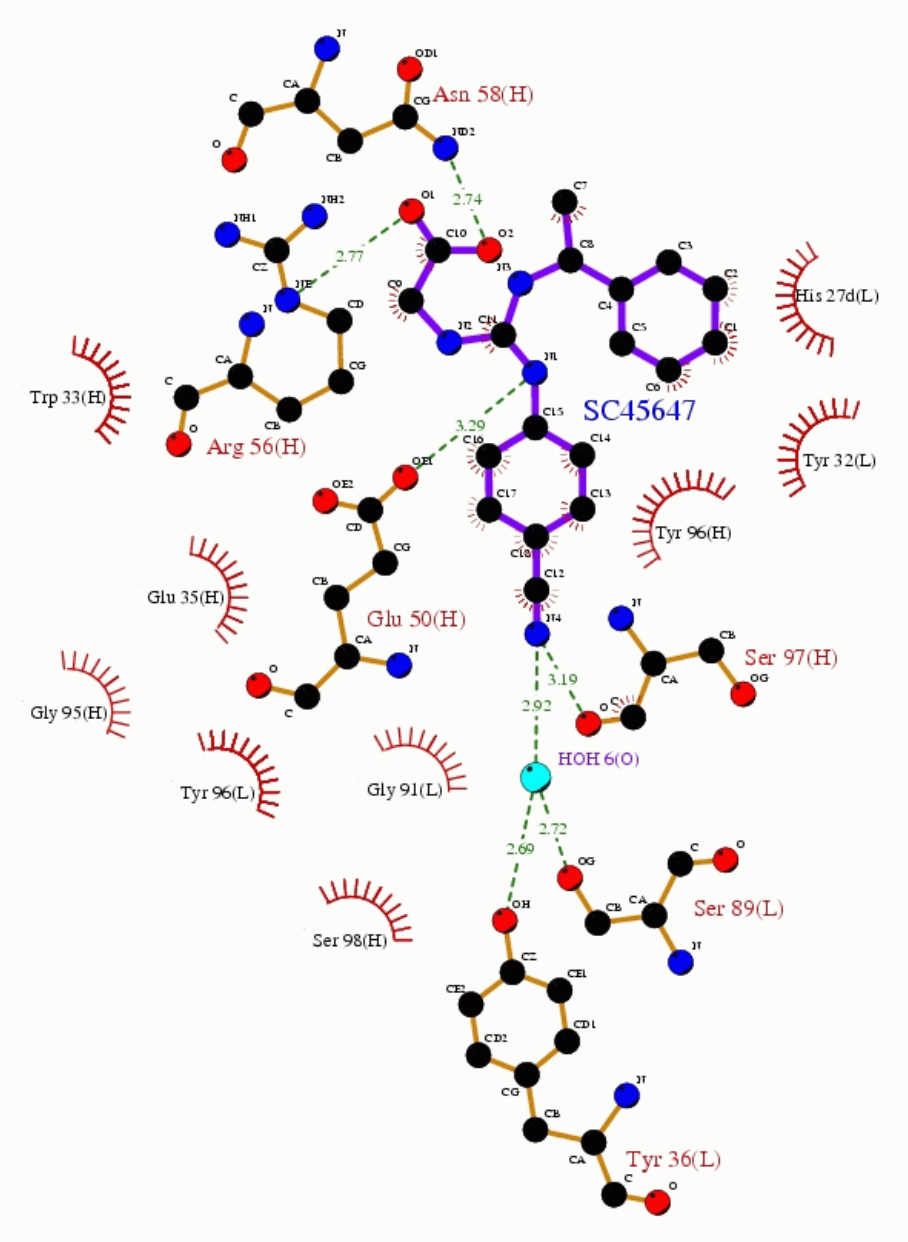

Figure 4a. Key interactions of high-potency sweetener SC45647 with NC6.8. Interaction patterns for the cyanophenyl and acetyl group are very similar to NC174, while the hydrophobic moieties are different and show different hydrophobic interactions. Note the presence of a chiral center at C8 (R conformation). Diagram created using *LIGPLOT* (Wallace et al., 1995). All *LIGPLOT* figures are oriented so that the ligands point down into the antigen binding pocket.

3.3 Binding of TES buffer

When co-crystallization of low affinity sweetener compounds NC90 (200 times sweeter than sucrose), NC24 (230x), NC274 (130xX) and Aspartame (150x) in 100-fold molar excess

with NC6.8 Fab antibodies was attempted, none of the low affinity sweetener compounds could be detected in the antigen-binding pocket. Clear electron density, however, was visible for TES protein stock buffer in the antigen-binding pocket (Figure 2b) instead of the expected low sweetener compounds in all co-crystal structures. Zwitterionic TES (N-[tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid) essentially consists of a electronegative sulfonyl group with a short, two carbon linker to a positively charged tris(hydroxymethyl) methyl-amino group.

The electronegative sulfonyl group of TES, located down in the antigen binding pocket, forms an extensive network of hydrogen bonds with residues of the antigen-binding pocket. In particular, the main chain amide hydrogens of Tyr 96H and Ser 98H, together with side chain interactions to His 34L and to Ser 89L via a tightly bound water atom, constitute a cluster of hydrogen bonded interactions that coordinate all three oxygen atoms of the sulfonyl group (Figure 4b). The tris(hydroxymethyl)methyl-amino group of TES is exposed to solvent and its positively charged N1 interacts via a water molecule with Ser 98H. The three hydroxy-methyl groups interact through a water mediated hydrogen bond network with both backbone and side chain atoms of numerous residues (Figure 4b). The positions of the respective carboxyl groups in both sweetener structures versus the sulfonyl group of TES do not coincide. Instead, the TES sulfonyl group occupies the approximate position of the CN group in the sweeteners (Figure 5), deep in the antigen binding pocket of NC6.8. In agreement with the chemistry of the zwitterionic ligand, there are only few hydrophobic interactions compared to the NC174 and SC45647 molecules.

In an attempt to still obtain low potency sweetener–Fab complexes, NC6.8 Fab was dialyzed against phosphate or Tris buffers instead of TES buffer. Unfortunately, attempts to crystallize NC6.8 in non-TES buffers with or without sweetener compounds have failed so far. We speculate that the TES may play an unexplained but critical role in the crystallization of the NC6.8 Fab under the conditions investigated.

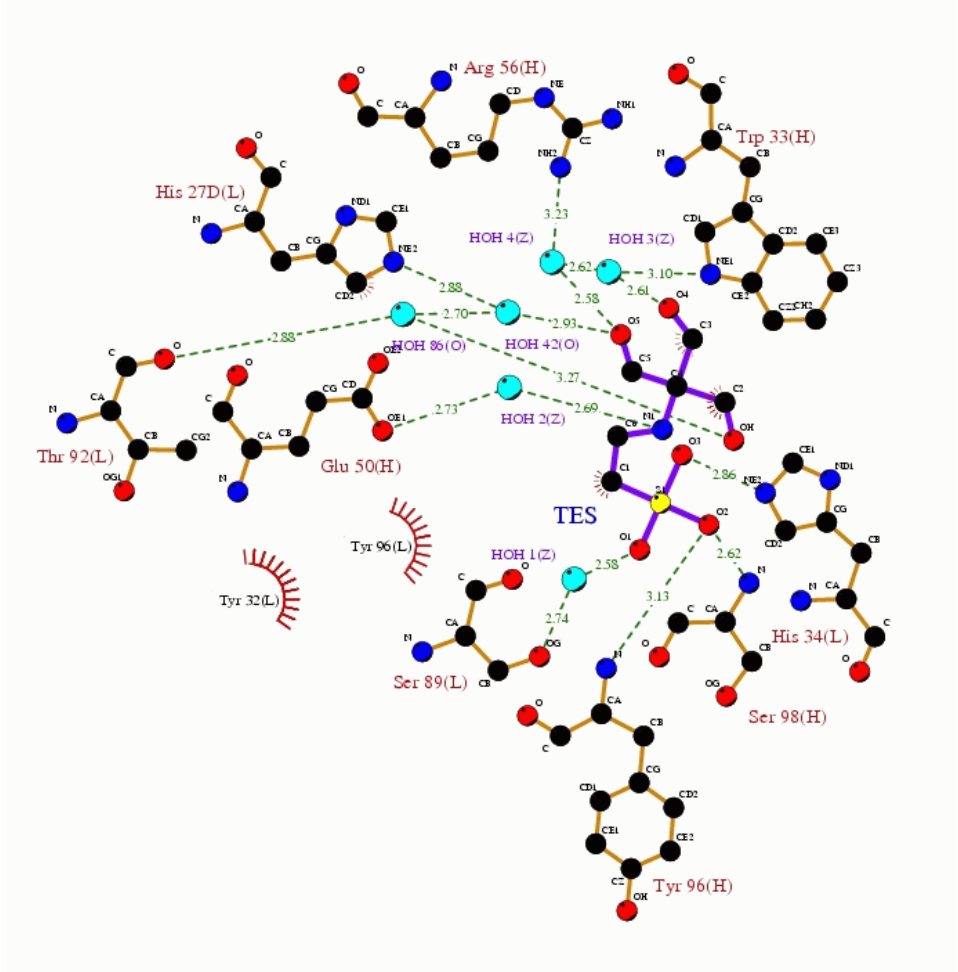

Figure 4b. Key interactions of non-sweetener TES with NC6.8. Compared to sweet tasting compounds NC174 and SC45647, the non-sweetener TES exhibits a significantly different binding pattern, dominated by a complex, water mediated hydrogen bond network and only few hydrophobic interactions.

4. Discussion

4.1 Structural comparison of the NC6.8-SC45647 and NC6.8-NC174 sweetener complexes

General. The sweetener ligands NC174, SC45647, as well as TES buffer, bind in the same antigenic pocket, formed by residues from CDR loops H1, H2, H3, L1 and L3 of the NC6.8 Fab fragment (Figure 4a-c). NC174 and SC45647 are trisubstituted guanidine tastants. Both synthetic sweeteners contain a distinct electronegative cyanophenyl group, a zwitterionic guanido core, an acetyl group and a large hydrophobic moiety. Substitution of one phenyl group in NC174 with a

methyl group in SC45647 creates a chiral center at C8, and causes a reduction of super-sweetener potency by nearly one order of magnitude. Although the NC6.8 monoclonal antibody was raised against the NC174 sweetener, it recognizes both NC174 and the R-stereoisomer of SC45647 in a similar fashion.

In both binary complexes, the cyanophenyl moiety occupies the same position (Figure5) and is deeply buried in the antigen binding pocket. As described in detail for SC45647, in both cases the cyanophenyl group is sandwiched between the light-chain and heavy-chain by p-stacking to Tyr 96L and stabilized by hydrogen bonds from the CN group via a conserved water molecule to Ser 98L and Tyr 36L. The possible role of several water molecules in the binding has been discussed for NC174 (Guddat et al., 1994). While we cannot confirm any specific conservation of other water molecules in the binding cavity, the deeply buried water atom bridging the CN group to Ser 89L O? and Tyr 36L O? is clearly visible in all tree NC6.8 sweetener complexes. This water atom, however, has not been observed in the NC10.14-NC174 complex (Guddat et al., 2000) and the general role of water mediated contacts in sweetener binding remains open until further sweetener complex structures become available.

The guanido and acetate groups are oriented in similar positions in both binary complex structures (Figure 5). The guanido group in NC174 exhibits a strong hydrogen bond to the H chain backbone via Tyr 96H O, an interaction absent in the SC45647 complex. The acetyl groups of both ligands exhibit similar hydrogen bonding patterns, but electron density for the SC45647 acetyl group does show indications for possible secondary conformations consistent with observed minor conformations of the Arg 56H side chain.

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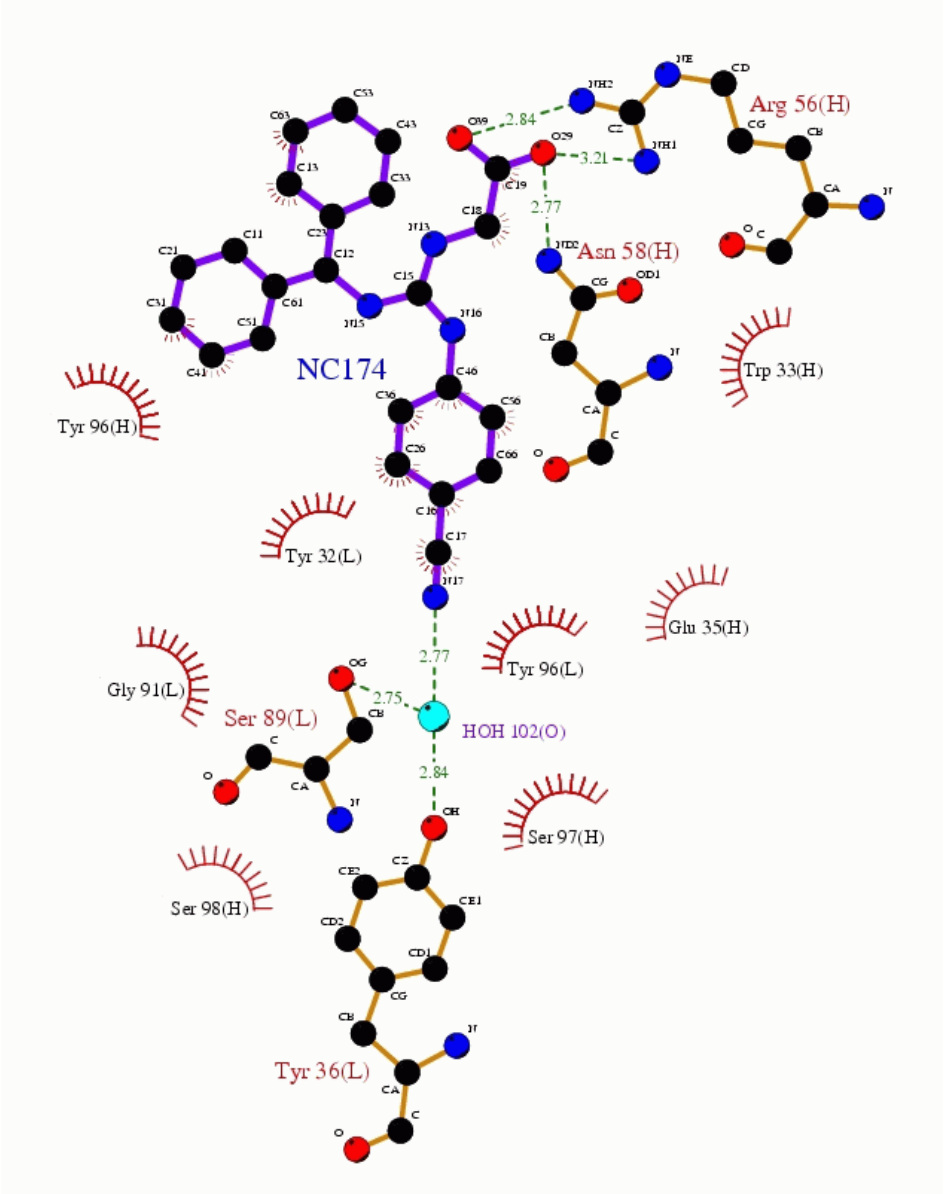

Figure 4c. Key interactions of super-potency sweetener NC174 with NC6.8. Interaction patterns for the cyanophenyl and acetyl group are very similar to SC45647 (figure 4a), while the larger hydrophobic biphenyl moiety shows stronger hydrophobic interactions with both the L and H chain of NC6.8). For clarity of the plot, a hydrogen bond from N15 to Tyr 96H backbone oxygen has been omitted. Residues from PDB entry 2CGR were renumbered to comply with Kabat nomenclature corresponding to figures 4a,b.

Role of the hydrophobic moiety in glucophore binding models. The structure comparison between Fab complexes suggests a significant role of the hydrophobic group in the interaction of the sweeteners with NC6.8. In 1967, a simple model was proposed (Shallenberger and Acree, 1967) stating that every sweetener compound has a hydrogen donor and a hydrogen acceptor

group, and the arrangement of the functional hydrogen bond donor and acceptor groups was thought to form anti-parallel hydrogen bonds with the receptor. A few years later it was suggested (Kier, 1972) that the presence of a third functional group, a hydrophobic moiety, is necessary for sweet-tasting molecules. The above hypothesis has been widely accepted, because of its ability to explain the sweetness of many structurally different compounds. In 1996, the multipoint attachment (MPA) theory for human taste receptors was proposed (Nofre et al., 1996). According to the MPA theory, human taste receptors should contain a minimum of eight binding (recognition) sites occupying the central cavity of the receptor, whose central cavity should be formed by Asp, Lys, Glu, Ser or Thr residues. Structural data from this study and others (Guddat et al., 2000, Guddat et al., 1994) indeed confirm that the antigenic epitope is surrounded by multiple charged residues, particularly at the bottom of the receptor binding site, and those residues actively participate in sweetener recognition.

As a result of different size and conformation of the hydrophobic groups of NC174 and SC45647, their specific interactions with residues of the antigenic epitope of NC6.8 vary. In NC174 (figure 4c) all of the three functional groups (cyanophenyl, tri-guanidino group and acetyl group, are positioned favorably to interact with residues of the antigenic epitope of NC6.8. The strong interaction and steric restraints reduce the freedom of rotation around the tri-guanidine group and restrict movement of the large biphenyl-substituted moiety of NC174. To accommodate the large biphenyl moiety of NC174, the antigen-binding pocket of NC6.8 widens compared to the SC45647 complex. In case of NC174, the phenyl group that corresponds to the single SC45647 phenyl ring interacts with both light-chain and heavy-chain residues, while the single phenyl ring of SC45647 interacts with only light-chain residues (also visible in Figure 3) and is probably less tightly bound as evidenced by slightly higher B factors. Differences in conformational freedom and composition of the hydrophobic moieties are thus very likely dominating factors in fine-tuning sweetener potency.

However, the extrapolation of details observed in binding of hydrophobic sweetener moieties in Fab complexes to their actual relevance in receptor binding needs to be treated with caution. The biphenyl group of NC174 in fact adopts different conformations in the Fab complex with NC6.8 (Guddat et al., 1994) and each of the two copies of NC10.14 (Guddat et al., 2000), which is likely the result of a reduced ability of NC10.14 to lock the biphenyl group in place (Guddat et al., 2000).

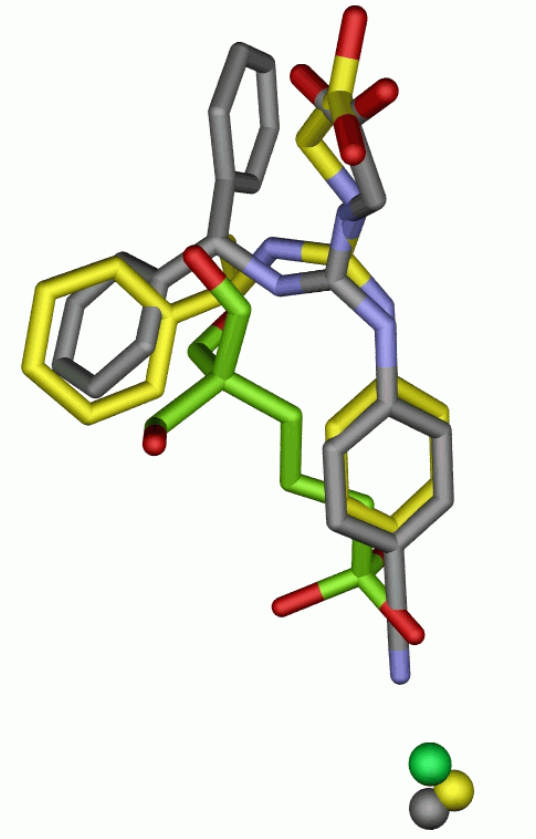

Figure 5. Ligands bound to NC6.8 antibody fragment. Superposition of ligands, oriented pointing down into the antigen binding pocket as in Figures 2 and 4. Grey C atoms: NC174, yellow C atoms: SC45647, green C and S atoms TES; the conserved water atoms participating in the hydrogen bonding to Ser 89L are colored correspondingly. Distinctly visible the similar positioning of the active groups in the sweeteners, with exception of the missing second phenyl group in SC45647, which lacks the additional contacts to heavy chain CDR residues. Superposition based on V domain residues VL1:107 and VH1:113 using LGA (Zemla, 2003), figure created with MSI LabViewer Lite and rendered with PovRay 3.5.

Conformational changes induced in the receptor molecule.

Upon binding of NC174, the NC6.8 Fab undergoes an unusually large allosteric conformational change, evidenced by a change in the Fab elbow angle by ~30 deg, which has led to reconsideration (Guddat et al., 1994) of the idea of elbow bending as a mechanism for signal transduction from the variable to the constant antibody domains. Although the evidence for such a mechanism in complete antibodies is sparse (Wilson and Stanfield, 1994), ligand induced conformational changes in the actual taste receptors are likely important, and the NC6.8-NC174 system has been extensively used in molecular dynamics modeling (Sotriffer et al., 2000).

Interestingly, no significant changes in elbow angle compared to free NC6.8 were calculated for either the high potency sweetener SC45647 nor the non-sweetener TES complex (Table 2), as also evidenced by a small overall backbone r.m.s.d. and visualized in figure 6. For NC6.8, the large change in elbow angle upon super-sweetener binding seems to be quite unique. Given that neither high potency sweetener SC45647 nor the non-sweetener TES induce a domain orientation change indicates that no simple correlation between sweetener potency and a possible allosteric effect in tastant binding exists, at least for NC6.8.

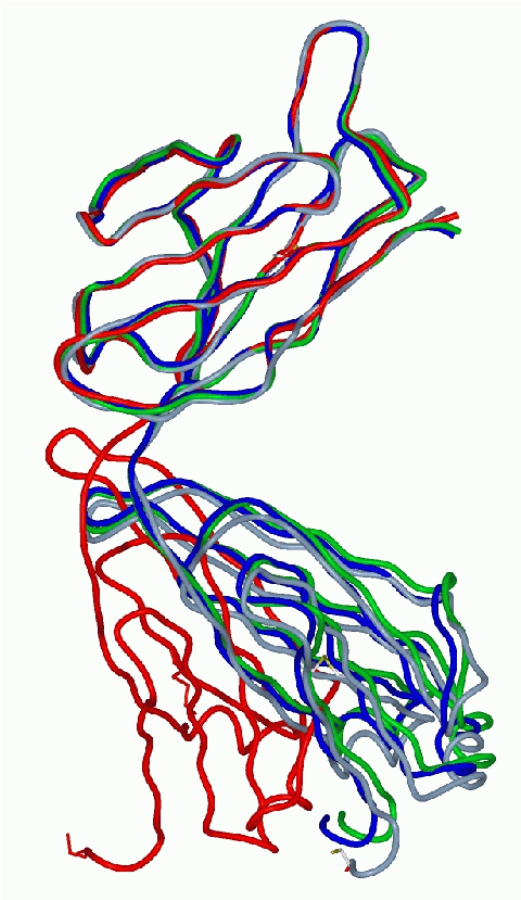

Figure 6. Elbow angles of complexed and free NC6.8. Shown are variable domain superpositions of the light chains of free NC6.8 (grey), NC174 (red), SC45647 (blue) and TES (green) complexes. The large change in elbow angle upon formation of the NC174 complex (red) is evident, and no significant changes in elbow angle upon complex formation with SC45647 and TES take place. Superposition on residues 1:107 using LGA (Zemla, 2003), figure rendered with ICM Pro (Abagyan et al., 1994).

Upon binding of the sweeteners, local conformation changes in the antigen binding pocket occur, as antigenic epitope residues shift to accommodate the ligands. In particular, Tyr 96H significantly moves from the free conformation and covers the binding pocket, while Trp 33H opens the pocket from the free position to accomodate the p-stacking with the guanido groups (Figure 7). Similar patterns of movement are also observed for residues His 27L, Arg 58H and Asn 56H. The remaining antigenic epitope residues participating in sweetener binding undergo only smaller displacements from their native conformation.

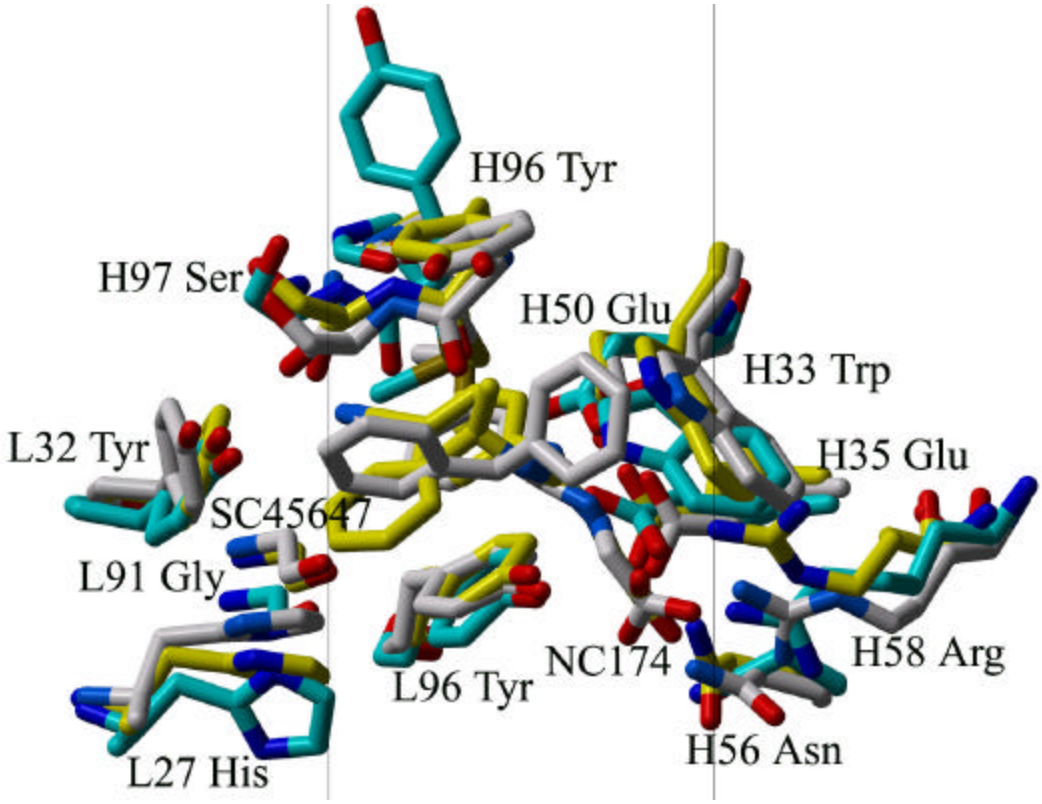

Figure 7. Conformation changes of key residues of NC6.8 upon NC174 and SC45647 binding. View down into the antigen binding pocket. Free NC6.8 C atoms displayed in cyan, complexed conformation and ligand C atoms in gey for NC174 and yellow for SC45647. Distinctly visible the large conformational change Tyr H56 covering the ligand binding pocket, and the reorientation of Trp H33 that p-stacks with the guanido group of SC45647 while Tyr 96H p-stacks with the phenyl moeity of SC45647. The water atoms bridging the CN groups to Ser 89L are buried deep at the bottom of in the antigen binding pocket and are not shown in this figure. Superposition on residues 1:107 using LGA (Zemla, 2003), figure rendered with ICM Pro (Abagyan et al., 1994).

4.2 Comparison between other sweetener and non-sweetener (TES) complexes

In the TES bound structure key residues Trp 33H, His 27L, Arg 56H as well as Glu 50H and Thr 92L interact with TES only indirectly via water molecules, whereas in NC174 and SC45647 complexes these residues interact either directly or via p-stacking and hydrophobic interactions with the respective sweetener compounds. In addition, no hydrophobic moiety is present in TES. Superposition of the NC6.8-TES complex with the other sweetener complexes reveals that the electronegative sulfonic acid group is located in a similar position as the electronegative cyanophenyl group (Figure 6), located deeply in the antigenic sweetener binding pocket. The water molecule mediating the interaction of the sweetener cyanophenyl groups to Ser 89L is conserved and links the sulfonyl group via O1 to the bottom of the antigen binding pocket.

Interestingly, this location of the sulfonyl group has in fact been predicted for a variety of sulfonyl-based high- to medium-potency sweeteners. Certain high potency sweeteners such as cyclamate (cyclohexylsulfamate) and its analogues (dihydrochalcone derivatives), contain sulfonic acid groups (DuBois et al., 1981). Sodium lauryl sulfate, a surfactant, has been reported to be a taste modifier which may act by disrupting the membrane of the taste buds (M.G., 1991, DuBois et al., 1981). Recent computational studies (Prakash et al., 2001) indicate that neotame and its modifiers such as cinnamic acid, various substituted benzoic acids, tyrosine and serine, adopt conformations similar to each other and act competitively at the taste receptors. Superposition of bound TES with the proposed active conformation of neotame in the receptor pocket reveals that both compounds assume similar overcall conformations (Figure 7), but the charged sulfonyl group of TES superimposes with hydrophobic t-butyl moiety of neotame. Although the amide groups superimpose closely, the details of the proposed neotame binding must differ from the ones observed in the sweetener complex, notably the absence of any charged interactions at the bottom of the antigen binding pocket.

Absent in TES is any hydrophobic moiety, which according to the model of (Kier, 1972) is necessary for sweet-tasting molecules. In order to test whether TES tastes sweet, a blind tasting of distilled water, 100 nM, 1 mM, 10 mM and 100 mM TES buffer (pH 6.0) was

conducted. At no concentration could any sweet taste be established, only a soapy and slightly sour taste perception at 10 and 100 mM, consistent with the proposed requirement of a hydrophobic moiety for sweet taste perception.

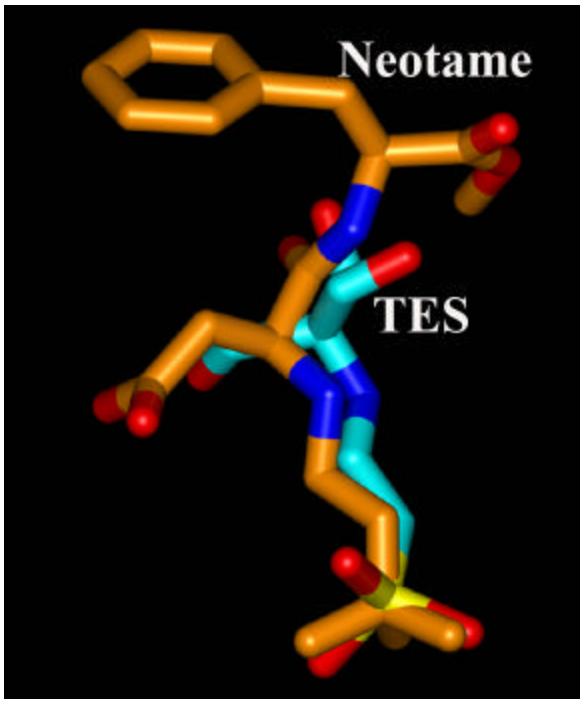

Figure 8. Superposition of TES bound to NC6.8 with the proposed active conformation of neotame. Same orientation as in Figures 2,4 and 6, ligands pointing into binding pocket at bottom. Overall, the geometry and position of the charged exhibits some similarity, but the position of the hydrophobic neotame t-butyl group coincides with the negatively charged TES sulfonyl group position determined experimentally in the NC6.8-TES structure.

Although TES effectively competes for Fab binding even in the presence of 100-fold excess of low potency sweetener compounds, is not able to compete with super and high potency sweeteners for NC6.8 binding. TES thus has significantly more affinity towards NC6.8 than low sweetener compounds, and can act as a sweet taste antagonist or a competitive inhibitor. Compared to the high affinity sweeteners, the conformational changes induced in NC6.8 upon TES binding are less pronounced than those induced by NC174 and SC45647 sweeteners, and as shown in figure 4b, most ligand - complex interactions are indirectly mediated through a complex (and likely more flexible) water network

Conclusions

The availibility of antibody-sweetener complexes ranging from super-potency tastants to high-potency (Guddat et al., 1994, Guddat et al., 2000) and non-sweetening compounds (this study) amplifies the complex nature of receptor interactions involved in sweet taste perception..While many details remain to be examined in further structural studies, a consistent picture begins to emerge in identifying key residues and interactions as well as the role of hydrophobic moieties in the molecular recognition of substituted tri-guanidine tastants.

Reflecting the chemical complexity of the tastants, the receptor-ligand interactions include a complex array of tight hydrogen bonds and charged interactions (Asn 58H, Arg 56H, Glu50H, Ser97H, Tyr 96H) and a significant number of hydrophobic contacts, with a substantial contribution of π-stacking (Trp33H, Tyr32L). It is very likely that the difference in super versus high potency guanidine sweeteners and related zwitterionic low-potency tastants is significantly determined by the nature and conformation of the hydrophobic moiety. The complex structure with non-tastant TES, which effectively competes against low-potency sweeteners, shows that although many of the key residues are involved in ligand binding, the interactions are largely indirectly mediate via water atoms. In addition, the zwitterionic antagonist TES does not possess ant hydrophobic group, emphasizing the necessity of a hydrophobic moiety in taste perception of tri-substituted, zwitterionic guanidine sweeteners. Interestingly, a conserved water molecule (bound to Ser 89L and Tyr 36L) buried deeply in the antigen binding pocket of NC6.8 is involved in the binding of all investigated molecules (super- potency NC174, high-potency SC45647 as well as the non-tastant TES), which seems to indicate a major role of the conserved water in ligand binding to NC6.8. The reported absence of the conserved water molecule in the NC10.14 complex with NC174 (Guddat et al., 2000) however means that its true role in taste perception remains open.

The absence of a any major conformational change of the Fab domain arrangement as monitored by the elbow angle for both high potency sweetener and non-tastant TES also rules out the necessity of a major allosteric rearrangement as a general prerequisite for ligand binding in NC6.8. It is still possible that a discrimination between super- and high-potency sweeteners may depend on the ability of the tastant to induce a large scale conformation change in the taste receptor. In absence of a taste receptor-sweetener structure, a free NC10.14 Fab structure might

establish whether a significant conformational change is a systematic feature in the discrimination of super versus high potency sweeteners, or a feature only found in the specific interaction of NC174 with the NC6.8 Fab (Guddat et al., 1994).

The structures confirm that sweetener potency is fine-tuned by multiple interactions between specific amino acid residues and the functional groups of the sweeteners. The identification of key interactions in structural studies is encouraging for attempts to design novel, high potency synthetic sweetener compounds. Since the antigen binding pocket of NC6.8 can adopt to multiple ligands - including high affinity antagonists - it is important that structure guided sweetener design based on available complexes carefully considers the exceptional structural adaptability of receptor-mimicking antibody models.

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Table 1. Data collection and refinement statistics for NC6.8-SC45 and NC6.8-TES complexes

Data Collection	**NC6.8-SC45**	**NC6.8-TES**
PDB ID code	1YNK	1YNL
Space Group	C2	C2
Wavelength (Å)	1.0000	1.0000
Temperature (Kelvin)	120	120
a(Å)	135.6	136.2
b(Å)	48.19	48.14
c(Å)	75.35	76.09
β (deg)	109.04	109.50
Resolution (Å)	23.64 - 2.1	23.25- 1.7
Highest resolution bin (Å)	2.2 - 2.1	1.75-1.7
Observed reflections [a)]	813664 (58014)	276375 (19682)
Unique reflections [a)]	24844 (2309)	43252 (2444)
% Completeness [a)]	92.7 (71.1)	85.0 (69.0)
R(merge) [a)]	0.07 (0.32)	0.03 (0.08)
$< I/s_{(I)} >$ [a)]	89.6 (2.8)	56.9 (10.9)
V_m (Matthews Coefficient)	2.43	2.46
% Solvent	49.4	49.9
Refinement		
Free R value , random, 5% [a]	0.277 (0.367)	0.241 (0.350)
R value	0.216 (0.272)	0.208 (0.232)
protein residues	438	438
water molecules	126	247
SC45647	1	0
TES	0	1
Rmsd bond length (Å)[b)]	0.027	0.015
Rmsd bond angle (Å) [b)]	2.459	1.828
Overall coordinate error (Å) [c)]	0.23	0.13
RSCC (*Shake&wARP*) [d)]	0.93	0.94
RSCC (*Refmac5*) [e)]	0.95	0.95
Ramachandran appearance [f)]		
Most favored (number, %)	329 (88.4)	337(90.6)
Allowed (number, %)	37(9.9)	29(7.8)
Generously allowed (number, %)	3 (0.8)	4 (1.1)
Disallowed (number, %)	3 (0.8)	2 (0.5)

[a] Values in parenthesis for the highest resolution bin

[b] Deviations from restraint targets (Engh and Huber, 1991)

[c] Estimated Standard Uncertainty, Diffraction Precision Index (DPI) based on R free (Cruickshank, 1999)

[d] Real Space Correlation Coefficient, F_c map against averaged and weighted *Shake&wARP* map (Reddy et al., 2003)

[e] Real Space Correlation Coefficient, F_o map against F_c map, as reported by *Refmac5* (Murshudov et al., 1997)
[f] Regions as defined in PROCHECK (Laskowski et al., 1993)

Table 2: Elbow angles of antibody sweetener complexes.

Crystal Structure	PDB code	Elbow Angle [a)]
NC6.8 (native)	1CGS	189
NC6.8-NC174	2CGR	152
NC6.8-SC45647	1YNK	188
NC6.8-TES	1YNL	190
NC10.14(LH)-NC174	1ETZ	193
NC10.14(AB)-NC174	1ETZ	192

[a)] All Fab elbow angles calculated consistently following the method of Wilson and Stanfield (Wilson and Stanfield, 1994) using LGA superpositions (Zemla, 2003). Absolute error estimate +/- 1.0 degrees. Angles published for 1ETZ (Guddat et al., 2000) are the complement angles.

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